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STANDARD ARTICLE

Lipid peroxidation biomarkers for evaluating oxidative stress in equine neuroaxonal dystrophy

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Background: Equine neuroaxonal dystrophy/equine degenerative myeloencephalopathy (eNAD/EDM) is a neurodegenerative disorder affecting genetically predisposed foals maintained on an α -tocopherol (α -TOH) deficient diet. Currently no antemortem diagnostic test for eNAD/EDM is available.

Hypothesis: Because α -TOH deficiency is associated with increased lipid peroxidation, it was hypothesized that F_2 -isoprostanes (F_2 IsoP), F_4 -neuroprostanes (F_4 NP) and oxysterols derived from free radical oxidation would be increased in the cerebrospinal fluid (CSF) and neural tissue of eNAD/EDM affected horses and could serve as potential biomarkers for disease.

Animals: Isoprostane Study A: 14 Quarter horse foals (10 healthy foals and 4 eNAD/EDM affected foals) at 1 and 6 months of age. Isoprostane Study B: 17 eNAD/EDM affected and 10 unaffected horses ≥ 1 -4 years of age. Oxysterol study: eNAD/EDM affected ($n = 14$, serum; $n = 11$, CSF; $n = 10$, spinal cord [SC]) and unaffected horses 1-4 years of age ($n = 12$, serum; $n = 10$, CSF; $n = 7$, SC).

Procedures: Cerebrospinal fluid [F_2 IsoP] and [F_4 NP] were assessed using gas chromatography-negative ion chemical ionization mass spectrometry. Serum, CSF, and cervical SC [oxysterols] were quantified using high performance liquid chromatography mass spectrometry. Results were compared with respective α -TOH concentrations.

Results: Spinal cord [7-ketocholesterol], [7-hydroxycholesterol], and [7-keto-27-hydrocholesterol] were higher in eNAD/EDM horses whereas [24-ketocholesterol] was lower. No significant difference was found in CSF [F_2 IsoP] and [F_4 NP], serum [oxysterols] and CSF [oxysterols] between eNAD/EDM affected and unaffected horses. No correlation was found between [F_2 IsoP], [F_4 NP], or [oxysterols] and respective [α -TOH].

Conclusions and Clinical Importance: In the SC, targeted markers of cholesterol oxidation were significantly increased in horses with eNAD/EDM.

KEYWORDS

ataxia, equine, genetics, vitamin E

Abbreviations: α -TOH, α -tocopherol; CI, confidence interval; CSF, cerebrospinal fluid; CYP, cytochrome P450; EDM, equine degenerative myeloencephalopathy; eNAD, equine neuroaxonal dystrophy; EtOH, ethanol; F_2 IsoP, F_2 -isoprostanes; F_4 NP, F_4 -neuroprostanes; HPLC-APCI-MS/MS, high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry; HPODE, hydroperoxy octadecadionoates; LC/MS, liquid chromatography/mass spectrometry; LXR, liver X receptor; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; QH, Quarter Horse; RRR- α -TP, natural (or -d) α -tocopherol; SC, spinal cord; SRM, selective reaction monitoring; TBAR, thiobarbituric acid reactivity; UHPLC-MS/MS, ultra-high performance liquid chromatography tandem mass spectrometry; VitE, vitamin E

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1 | INTRODUCTION

Equine neuroaxonal dystrophy/equine degenerative myeloencephalopathy (eNAD/EDM) is a neurologic condition that develops in genetically predisposed foals maintained on an α -tocopherol (α -TOH) deficient diet.^{1,2} The disease appears to develop during the first year of life³ and, in humans, there is strong evidence that the developing nervous system is particularly at risk from α -TOH deficiency.⁴ The underlying genetic cause remains unknown. Although α -TOH deficiency in the cerebrospinal fluid (CSF) may be supportive of the diagnosis in younger horses (ie, <6 months of age),⁵ no definitive antemortem diagnostic test currently is available. Decreased CSF α -TOH concentrations in horses > 6 months of age may not be specific for eNAD/EDM.

The term vitamin E (vitE) encompasses a closely related family of 8 fat soluble naturally occurring compounds. Of these forms, α -TOH most consistently prevents lipid peroxidation *in vitro*⁶ and *in vivo*.^{7,8} Biomarkers of lipid peroxidation often are assessed as indicators of oxidative damage. Potential biomarkers include F₂-isoprostanes (F₂IsoP), formed by oxidation of arachidonic acid and F₄-neuroprostanes (F₄NP) from docosahexaenoic acid.⁹ The measurement of lipid peroxidation products is more reliable than the detection of reactive oxygen species, reactive nitrogen species, and other active oxidants.¹⁰

Lipid peroxidation products have various biological functions *in vivo* such as regulating gene expression and cell signaling.^{11,12} In eNAD/EDM affected horses, we recently identified upregulation of specific genes targeted by a nuclear transcription factor, the liver X receptor (LXR).¹³ The natural ligands for LXR activation are oxysterols.^{14,15} Preliminary evaluation of eNAD/EDM affected horses identified a trend toward increased spinal cord (SC) 7-ketocholesterol and 7-hydroxycholesterol concentrations.¹³ These 2 oxysterols result from autooxidation of cholesterol and oxidation of 7-dehydrocholesterol by cytochrome P450 7A1 (CYP7A1; 7- α -hydroxylase).¹⁶

We hypothesized that measures of lipid peroxidation, including F₂IsoP, F₄NP, and oxysterols would be significantly increased in the CSF of eNAD/EDM affected horses. Additionally, we hypothesized that the same oxysterol markers would be increased in SC tissue of eNAD/EDM affected horses. The purpose of our study was to determine the concentrations of these biomarkers in eNAD/EDM affected horses to potentially provide an antemortem diagnostic tool while further investigating the etiology of the disease.

TABLE 1 Sample sizes used for assays in this study

Analysis	Age	Analytes	Sample	Horses	
				#Affected	#Unaffected
Isoprostane Study A	Repeated samples 1 and 6 mo	F ₂ -isoprostane and F ₄ -neuroprostanes	CSF	4	10
Isoprostane Study B	1–4 y	F ₂ -isoprostane and F ₄ -neuroprostanes	CSF	17	10
Oxysterol	1–4 y	Cholesterol and oxysterols	Serum	14	12
Oxysterol	1–4 y	Cholesterol and oxysterols	CSF	11	10
Oxysterol	1–4 y	Cholesterol and oxysterols	SC	10	7

For a full list of individual horses, refer to Supporting Information Table S1.

Abbreviations: CSF, cerebrospinal fluid; SC, spinal cord; mo, months; y, years.

2 | MATERIALS AND METHODS

2.1 | Animals

2.1.1 | Isoprostane study A: Foal samples

For repeat measurements of F₂IsoP and F₄NP at 1 and 6 months of age, 14 Quarter horse foals were used as previously described (Table 1).⁵ These included 4 foals that developed eNAD/EDM during the first year of life, which was confirmed on postmortem examination at 1.5 years, and 10 healthy control foals that were maintained in the same environment and fed an identical α -TOH deficient diet. Postmortem evaluation of the brain and SC tissue from eNAD animals indicated histologic lesions typical of eNAD, namely chromatolytic neurons and spheroid development that were especially prominent in the lateral accessory cuneate, medial cuneate, and gracile nuclei. Serum and CSF samples were collected as previously described.⁵ All protocols were approved by the UCD Institutional Animal Care and Use Committee (Protocol # 15866).

2.1.2 | Isoprostane study B: Adult samples

Over a period of 8 years, samples from 19 postmortem-confirmed eNAD/EDM and 16 unaffected horses were collected (Supporting Information Table S1). All horses were donated by owners for the purposes of the study and were between 1 and 4 years of age. Biologic samples (ie, serum, CSF, and SC tissue) were available on subsets of eNAD/EDM affected and unaffected horses (Table 1 and Supporting Information Table S1) and collected immediately before (serum) or after (CSF, SC tissue) euthanasia. A moderate phenotype (eNAD) or severe phenotype (eNAD/EDM) was diagnosed using histologic lesions as previously described.² Unaffected horses were euthanized for reasons other than neurologic disease and subclinical eNAD was excluded by histologic evaluation of the central nervous system.

2.1.3 | Oxysterol study

Samples from n = 14 (serum), n = 11 (CSF), and n = 10 (SC) eNAD/EDM affected and n = 12 (serum), n = 10 (CSF), and n = 7 (SC) unaffected horses ages 1–4 years were available for oxysterol quantification (Table 1 and Supporting Information Table S1). Spinal cord sections were collected at the level of C1 as previously described.¹³

2.2 | Sample collection and storage

Cerebrospinal fluid (10–12 mL) was aliquoted into plastic light-protected vials on ice and centrifuged at 4°C (2000g for 15 minutes) within 1 hour of collection. Supernatant was aliquoted 1 mL at a time into precooled cryovials, immediately flash frozen in liquid nitrogen and maintained at –80°C until analysis. Serum samples were collected from an IV catheter after removal of 6 mL of heparinized blood. Serum samples were processed in an identical manner as CSF and frozen in 1 mL aliquots. For F₂IsoP, F₄NP, and oxysterol quantification, samples were shipped to the respective laboratories overnight on dry ice.

2.3 | α -TOH concentrations

For isoprostane Study A, α -TOH concentrations were assessed in serum and CSF from foals at 1 and 6 months of age as previously described.⁵ In Isoprostane Study B and oxysterol studies, α -TOH concentrations were assessed in serum (n = 19, eNAD/EDM affected; n = 8, unaffected), CSF (n = 15, eNAD/EDM affected; n = 7, unaffected), and SC tissue (n = 5, eNAD/EDM affected; n = 6, unaffected) as previously described (Supporting Information Table S1).¹³

2.4 | F₂-isoprostane and F₄-neuroprostane concentrations

F₂-isoprostane (F₂-IsoP, also referred to as 8-iso-PGF_{2 α}) and F₄-NP concentrations were assessed in CSF from horses in Study A at 1 and 6 months of age and in Study B at the time of euthanasia (1–4 years of age). Quantification of F₂IsoP and F₄NP was performed using a gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) approach employing stable isotope dilution as previously described.¹⁷ The limit of detection for both assays was 1 pg.

2.5 | Cholesterol and oxysterol concentrations

Selected oxysterol concentrations were assessed based on preliminary data indicating detectable concentrations in biologic samples.¹³ Serum 7-ketocholesterol, 7-hydroxycholesterol, 24-hydroxycholesterol, and 4 β -hydroxycholesterol; CSF 7-ketocholesterol and 24-epoxycholesterol; and SC tissue 7-ketocholesterol, 7-hydroxycholesterol, 24-hydroxycholesterol, 24-ketocholesterol, 7-keto-27-hydroxycholesterol, and 7-27-dihydroxycholesterol concentrations were quantified.

2.5.1 | Materials

Liquid chromatography/mass spectrometry (LC/MS) grade solvents (methylene chloride, chloroform, methanol, water, formic acid) were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, Massachusetts). The d₇-7-ketocholesterol was prepared as reported previously.¹⁸ Two-hundred proof ethanol (USP Specification; Decon Laboratories, King of Prussia, Pennsylvania), potassium hydroxide (ACS certified; Thermo Fisher Scientific, Waltham, Massachusetts), and sodium chloride (ACS certified; Thermo Fisher Scientific, Waltham, Massachusetts) were used.

2.5.2 | Lipid extraction from tissues and fluids

Before lipid extraction, the internal standards, d₇-7-ketocholesterol (500 ng), d₆-24-epoxycholesterol (500 ng), and d₇-cholesterol (2.5 μ g) were added to each sample. For SC tissue, the tissues were homogenized in Folch solution (4 mL, chloroform : methanol = 2 : 1) by a blade homogenizer. For serum (100 μ L), 4 mL of Folch solution was added directly to each sample. Sodium chloride aqueous solution (0.9%, 1 mL) then was added, and the resulting mixture was briefly vortexed and centrifuged for 5 minutes. The lower organic phase was recovered and dried at room temperature using a SpeedVac (Thermo Fisher Scientific, Waltham, Massachusetts), and then re-dissolved in methylene chloride (1 mL for tissue samples and 500 μ L for CSF samples). For serum samples, extracts were dried under SpeedVac and the dried samples then were reconstituted in 2 mL 2.5% KOH in 90% ethanol (EtOH) and incubated in a water bath at 55°C for 55 minutes. Another lipid extraction was performed on the solution from the base hydrolysis and the extracts were dried under speed vacuum (Savant SpeedVac, Thermo Fischer Scientific, Waltham, Massachusetts). Each sample was reconstituted in methylene chloride (500 μ L).

2.5.3 | High-performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry (HPLC–APCI–MS/MS) analyses of sterols and oxysterols

Analysis of cholesterol and oxysterols was performed by ultra-HPLC tandem mass spectrometry (UHPLC–MS/MS) using a triple quadrupole mass spectrometer (API 4000, Sciex, Framingham, Massachusetts) equipped with APCI. For analysis, an appropriate amount of sample was transferred to a LC vial, dried under a stream of argon, and reconstituted in 90% methanol with 0.1% formic acid (for oxysterol analysis of SC tissue, 50 μ L was reconstituted in 200 μ L; for serum, 100 μ L to 50 μ L and for CSF, 100 μ L to 50 μ L). For cholesterol analysis, 20 μ L was reconstituted in 200 μ L for SC tissue, serum, and CSF. Reverse-phase chromatography was performed under the following conditions: C18 column (1.7 μ m, 100 mm by 2.1 mm; Kinetex, Phenomenex, Torrance, California); flow rate, 0.4 mL/min and elution solvent, 90% methanol with 0.1% formic acid. Mass spectroscopy conditions were nebulizer current, 3 mA; curtain gas, 10 psi; collision gas, high; ion source gas, 20 pounds per square inch; entrance potential, 10 V; collision energy, 25 V; declustering potential, 80 V; collision cell exit potential, 20 V and temperature, 300°C. For MS analysis, selective reaction monitoring (SRM) was employed to monitor the dehydration process of the ion [M + H]⁺ or [M + H–H₂O]⁺ as described previously.¹³ The internal standard, d₇-7-ketocholesterol, was used to quantify the analytes 7-ketocholesterol, 4 β -hydroxycholesterol, and 7-hydroxycholesterol. d₆-24-epoxycholesterol was used to quantify 24-epoxycholesterol, 24-ketocholesterol, and 24-hydroxycholesterol; and, d₇-cholesterol was used to quantify cholesterol. Selective reaction monitoring mass transitions were d₇-7-ketocholesterol *m/z* 408.3 \rightarrow 390.3; d₆-24-epoxycholesterol *m/z* 389.3 \rightarrow 371.3; 7-ketocholesterol, *m/z* 401.3 \rightarrow 383.3; 7-hydroxycholesterol, 4 β -hydroxycholesterol, and 24-hydroxycholesterol, *m/z* 385.3 \rightarrow 367.3; 24-epoxycholesterol and 24-ketocholesterol *m/z* 383.3 \rightarrow 365.3; d₇-cholesterol *m/z* 376.3 \rightarrow

376.3; cholesterol m/z 369.3 \rightarrow 369.3. All concentrations were normalized to cholesterol concentrations for quantification.

2.6 | Statistical analyses

2.6.1 | Isoprostane study A

A repeated measures 2-way ANOVA with Sidak's multiple comparisons was performed on F_2 IsoP concentrations. Because all F_4 NP results were below the limit of detection at 6 months of age, a Mann-Whitney test was used to compare values at 1 months of age.

2.6.2 | Isoprostane study B and oxysterol analyses

Data for each metabolite were analyzed for normality using a Shapiro-Wilk test. Normally distributed data were analyzed using an unpaired T-test with Welch's correction and non-normally distributed data with a Mann-Whitney test. Correction for multiple testing was performed at a false discovery rate of 0.1 within each sample subset.

2.6.3 | Correlation with age and α -TOH concentrations

Correlations of each metabolite with age and corresponding α -TOH concentrations (serum, CSF and SC tissue) were performed using a Pearson correlation for normally distributed data and a Spearman's rank correlation for non-normally distributed data. Analyses were performed using commercial software (GraphPad Prism7, La Jolla, California). Values are reported as mean \pm SD and $P < .05$ was considered significant.

3 | RESULTS

3.1 | F_2 -isoprostane and F_4 -neuroprostane concentrations

3.1.1 | Isoprostane study A

In the foal population (1–6 months of age), no effect of disease ($P = .42$) or age ($P = .92$) was found on CSF F_2 IsoP. The F_4 NP were below the limit of detection in $n = 2$ eNAD/EDM affected and $n = 3$ unaffected foals at 1 months of age. No significant effect of disease was found on CSF F_4 NP ($P = .91$).

3.1.2 | Isoprostane study B

A total of $n = 17$ eNAD/EDM affected (6 geldings, 2 stallions, 9 mares) and $n = 10$ unaffected (2 geldings and 8 mares) samples were available for analysis of CSF F_2 IsoP and F_4 NP from adult horses (Table 1 and Supporting Information Table S1). No correlation of CSF F_2 IsoP concentrations with age was found ($R^2 = 0.09$) and no significant difference in age between experimental groups was found (eNAD/EDM = 2.09 ± 1.03 years, unaffected = 2.77 ± 1.13 years; $P = .12$). No significant effect of disease on CSF F_2 IsoP in horses aged ≥ 1 –4 years ($P = .93$) was identified. All CSF F_4 NP were below the limit of detection.

3.1.3 | Correlation with CSF α -TOH

For both Studies A and B, no significant correlation was found between CSF F_2 IsoP and CSF α -TOH concentrations ($R^2 = 0.12$, $P = .22$, and $R^2 = 0.003$, $P = .82$, respectively).

3.2 | Cholesterol and oxysterol concentrations

3.2.1 | Serum

Serum samples were available from $n = 14$ eNAD/EDM affected (6 geldings, 2 stallions, 6 mares) and $n = 12$ unaffected (3 geldings, 1 stallion, 8 mares) horses. No correlation of serum 7-ketocholesterol, 7-hydroxycholesterol, 24-hydroxycholesterol, and 4 β -hydroxycholesterol concentrations with age was found and no significant difference in age between experimental groups (eNAD/EDM = 1.93 ± 0.96 years, unaffected = 2.37 ± 1.43 years; $P = .37$) was identified. In serum, no effect of disease on serum 7-ketocholesterol ($P = .23$), 7-hydroxycholesterol ($P = .32$), 24-hydroxycholesterol ($P = .53$), or 4 β -hydroxycholesterol ($P = .16$) concentrations was found.

3.2.2 | Correlation with serum α -TOH

Serum α -TOH concentrations were available from $n = 17$ of the 26 samples ($n = 12$ eNAD/EDM and $n = 5$ unaffected horses, Supporting Information Table S1). No correlation was identified between any serum oxysterol assessed and serum α -TOH concentrations.

3.2.3 | CSF

A total of $n = 11$ eNAD/EDM affected (4 geldings, 2 stallions, 5 mares) and $n = 10$ unaffected (3 geldings, 7 mares) horses were available for analysis of CSF oxysterols. No correlation of CSF 7-ketocholesterol or 24-epoxycholesterol concentrations was found with age and no significant difference in age between experimental groups (eNAD/EDM = 1.73 ± 0.72 years, unaffected = 2.4 ± 1.05 years; $P = .11$) was identified. In CSF, nonsignificantly ($P = .07$) increased 7-ketocholesterol concentrations were observed in eNAD/EDM affected horses (Supporting Information Fig. S1). No effect of disease on CSF 24-epoxycholesterol concentrations was found ($P = .99$).

3.2.4 | Correlation with CSF α -TOH

Cerebrospinal fluid α -TOH concentrations were available from $n = 16$ of the 21 samples ($n = 11$ eNAD/EDM and $n = 5$ unaffected horses). No correlation was found between any CSF oxysterol and CSF α -TOH concentrations.

3.2.5 | Spinal cord

Spinal cord samples were available from $n = 10$ eNAD/EDM affected (4 geldings, 1 stallion, 5 mares) and $n = 7$ unaffected (2 geldings, 2 stallions, 3 mares). No correlation of SC 7-ketocholesterol, 7-hydroxycholesterol, 24-hydroxycholesterol, 24-ketocholesterol, and 7,27-dihydroxycholesterol concentrations was found with age. A significant negative correlation was observed between age and 7-keto-27-hydroxycholesterol concentrations (Spearman $r = -0.02$ (confidence interval -0.73 – 0.04 , $P = .03$). No significant difference in

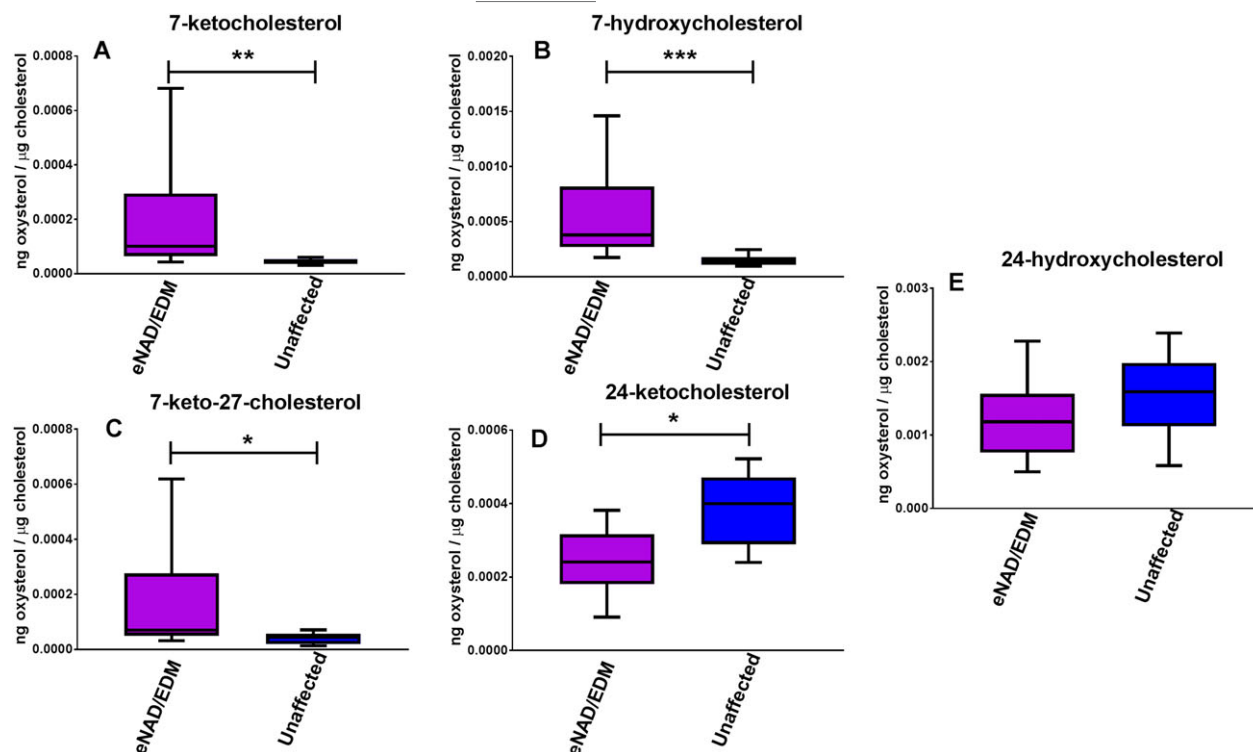


FIGURE 1 Spinal cord (A) 7-ketocholesterol, (B) 7-hydroxycholesterol, (C) 7-keto-27-hydroxycholesterol, (D) 24-ketocholesterol, and (E) 24-hydroxycholesterol concentrations from eNAD/EDM affected and unaffected horses. Spinal cord 7-ketocholesterol, 7-hydroxycholesterol, and 7-keto-27-hydroxycholesterol concentrations were significantly higher in eNAD/EDM affected horses while 24-ketocholesterol concentrations were significantly lower. Data as min to max, with box extending from 25th to 75th percentiles and line representing the median. * $P < .05$, ** $P < .01$, *** $P < .001$

age was found between experimental groups (eNAD/EDM = 1.35 ± 0.34 years, unaffected = 1.18 ± 0.25 years; $P = .48$).

Spinal cord 7-ketocholesterol ($P = .002$), 7-hydroxycholesterol ($P = .0007$), and 7-keto-27-hydroxycholesterol ($P = .03$) concentrations were significantly higher in eNAD/EDM affected horses (Figure 1A–C) whereas 24-ketocholesterol was significantly lower ($P = .02$; Figure 1D), even after accounting for multiple testing. No effect of disease on SC 24-hydroxycholesterol (Figure 1E) and 7,27-dihydroxycholesterol concentrations was identified. The majority (16/17) of SC tissue 7,27-dihydroxycholesterol concentrations were undetectable at the limit of detection across the sample set.

3.2.6 | Correlation with CSF α -TOH

Cerebrospinal fluid α -TOH concentrations were available from $n = 9$ of the 17 samples ($n = 3$ eNAD/EDM and $n = 6$ unaffected horses). No correlation was found between any SC oxysterol and SC α -TOH concentrations.

4 | DISCUSSION

Markers of lipid peroxidation, including F_2 IsoP, F_4 NP, and oxysterols, did not significantly differ in serum and CSF of eNAD/EDM affected and unaffected horses. However, in SC tissue, targeted markers of cholesterol oxidation, including 7-ketocholesterol, 7-hydroxycholesterol, and 7-keto-27-hydroxycholesterol were significantly increased with eNAD/EDM. These particular oxysterols are

formed by reactive oxygen species-mediated oxidation of cholesterol (Figure 2).¹⁶ 7-Hydroxycholesterol consists of both 7α -hydroxycholesterol, which is produced by CYP7A1 and free radical oxidation, and 7β -hydroxycholesterol, formed by free radical oxidation. Although CYP7A1 is not typically expressed in mammalian SC tissue, we recently identified significant upregulation of this transcript in eNAD/EDM affected horses.¹³ It is therefore possible that the increased SC 7-hydroxycholesterol concentrations are because of a combination of increased 7α -hydroxycholesterol from free radical oxidation and CYP7A1 enzymatic activity and that increased 7β -hydroxycholesterol is a consequence of free radical oxidation. Increases in 7-ketocholesterol and its metabolite, 7-keto-27-hydroxycholesterol, support further free radical oxidation of cholesterol in SC tissue of eNAD/EDM affected horses (Figure 2).

The main mechanism for removing cholesterol from the brain is enzymatic conversion of cholesterol to 24(S)-hydroxycholesterol through CYP46A1 (Figure 2). A shift toward non-enzymatic, free-radical mediated oxidation of cholesterol in the SC tissue of eNAD/EDM affected horses (Figure 1E) indicates that oxidative stress may contribute to disease pathogenesis. Alpha-TOH prevents lipid peroxidation⁶ and therefore the associated α -TOH deficiency observed with eNAD/EDM may directly lead to increased cholesterol oxidation in the central nervous system. However, no direct correlation was observed between SC α -TOH and oxysterol concentrations. We previously found that although serum, CSF and hepatic α -TOH concentrations are significantly lower in eNAD/EDM affected horses and highly correlated, SC tissue α -TOH concentrations did not differ and were

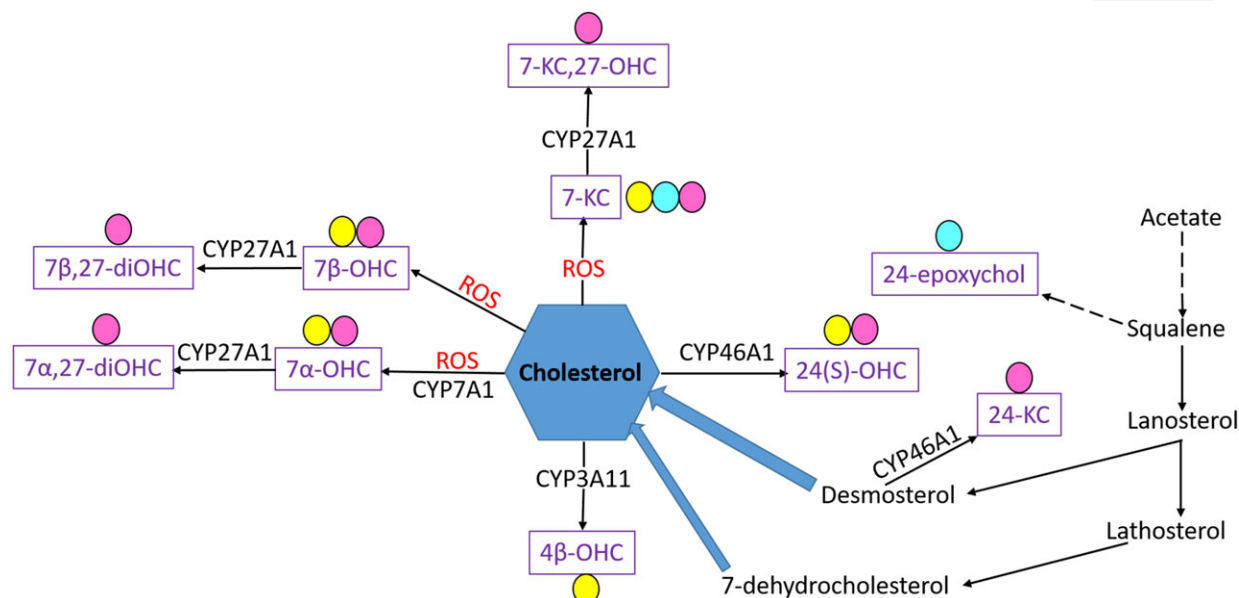


FIGURE 2 Mechanisms of cholesterol production and generation of oxysterols (purple font) via enzymatic and nonenzymatic oxidation. Specific oxysterols were detectable in equine serum (yellow circle), CSF (teal circle) and SC tissue (pink circle). Cyp, cytochrome P450; ROS, reactive oxygen species; 7-KC, 7-ketocholesterol; 7-KC,27-OHC, 7-keto-27-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; 7β,27-diOHC, 7β,27-dihydroxycholesterol; 7α-OHC, 7α-hydroxycholesterol; 7α,27-diOHC, 7α,27-dihydroxycholesterol; 4β-OHC, 4β-hydroxycholesterol; 24(S)-OHC, 24(S)-hydroxycholesterol; 24-KC, 24-ketocholesterol; 24-epoxychol, 24-epoxycholesterol

not correlated with serum concentrations.¹³ Therefore, although the pathology associated with eNAD/EDM is confined to the central nervous system and CSF appears to be an appropriate sample to use for assessment of α -TOH deficiency, SC tissue α -TOH concentrations may not reflect the deficiency to the same extent. This may be because of variable lipid concentrations in SC tissue, because lipid has the highest concentration of α -TOH.¹⁹ In addition, in the horse, cholesterol may be oxidized to oxidation products not included in our analysis.

The finding of increases in SC 7-ketocholesterol and 7-hydroxycholesterol concentrations in eNAD/EDM affected horses agrees with results reported previously. Our previous study identified increased expression of LXR-responsive transcripts, including CYP7A1 and APOE, in the SC tissue of eNAD/EDM affected horses.¹³ The liver X receptor is a nuclear transcription factor that requires activation by binding of an oxysterol ligand. The main ligands of LXR include 24(S)-hydroxycholesterol, 24,25-epoxycholesterol, 22-hydroxycholesterol, 20-hydroxycholesterol and 27-hydroxycholesterol.²⁰ Of these, only 24-hydroxycholesterol was quantified in the current study and found to not be increased in the SC tissue of eNAD/EDM affected horses. Therefore, our findings may represent downstream consequences after LXR activation rather than evidence of the initial ligand activation.

24-Ketocholesterol was significantly decreased in the SC tissues of eNAD/EDM affected horses. Although a negative correlation was observed with age and 24-ketocholesterol, similar to reports in other species,²¹ no significant difference in age was found between diseased and healthy groups. 24-ketocholesterol is produced by CYP46A1 from desmosterol (Figure 2).²² The enzyme CYP46A1 also is responsible for converting cholesterol to 24(S)-hydroxycholesterol, which was lower, but not significantly, in eNAD/EDM affected horses

(Figure 1E), potentially providing further support of decreased CYP46A1 activity. A similar phenomenon was observed by our group in mice deficient in tocopherol (alpha) transfer protein (*Ttpa*^{-/-}). These mice develop a postnatal sensory neurodegeneration like that observed in eNAD/EDM.²¹ In wild-type mice, concentrations of 24(S)-hydroxycholesterol significantly increase in the SC tissue with age, suggesting adequate CYP46A1 activity. In contrast, concentrations of 24(S)-hydroxycholesterol do not increase with age in *Ttpa*^{-/-} mice. Therefore, the enzymatic removal of cholesterol in SC tissue may be altered both in *Ttpa*^{-/-} mice and eNAD/EDM affected horses during postnatal development. Because various cholesterol products differ in lipophilicity and therefore are eliminated differentially across the blood brain barrier; this situation may contribute to the pathophysiology of α -TOH deficient neurodegeneration.

Paradoxically, despite higher SC 7-ketocholesterol concentrations in eNAD/EDM affected horses, CSF 7-ketocholesterol concentrations were non-significantly lower (Supporting Information Fig. S1). Like cholesterol, oxysterols can only be eliminated directly from cells to lipophilic acceptors, mediated by specific membrane lipid transporters, including ATP-binding cassette, subfamily A, member 1 (ACBA1); ATP-binding cassette, subfamily G, member 1 (ABCG1); and, scavenger receptor BI (SRBI).²³ Alterations in these proteins could interfere with oxysterol elimination from cells. Therefore, these are strong candidate genes for eNAD/EDM. Alternatively, altered oxysterol concentrations in neuronal plasma membranes may alter basic membrane properties.²⁴ Although lower CSF 7-ketocholesterol concentrations in eNAD/EDM affected horses warrants further evaluation in a larger sample set, this finding may suggest an altered transport mechanism for 7-ketocholesterol removal in eNAD/EDM.

F₂IsoP, F₄NP, and oxysterols are only a subset of markers used to assess lipid peroxidation in biologic systems. Additional markers, including malondialdehyde, which is one of the products formed from decomposition of certain primary and secondary lipid peroxidation products, and thiobarbituric-acid-reactivity have been used in other disease processes as markers of lipid peroxidation.⁹ However, these markers are not recommended for use in biologic systems without correlative data from other indices of fatty peroxide formation and decomposition.²⁵ Therefore, isoprostanes and oxysterols were selected for evaluation in our study. Limitations of our study include inability to perform all assays on all sample types and the lack of matched serum, CSF, and SC tissue results by individual. Additionally, because of limited sample availability, F₂IsoP and F₄NP were not evaluated in SC tissue. It is therefore possible that, similar to targeted oxysterols, F₂IsoP and F₄NP may be increased in SC tissue of eNAD/EDM affected horses without increased concentrations in the CSF.

In conclusion, targeted markers of cholesterol oxidation were significantly increased in the SC tissue of eNAD/EDM affected horses. Although serum and CSF biomarkers of eNAD/EDM were not identified, the mechanism of neurodegeneration was further elucidated. Enzymatic removal of cholesterol in SC tissue may be altered in eNAD/EDM affected horses during postnatal development, which could interfere with neuronal membrane signaling mechanisms, resulting in subsequent neurodegeneration.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All animal procedures were approved by the University of California-Davis IACUC and owner consent was obtained for all horses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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